Human defensin polypeptide Def-X, genomic DNA and cDNA, composition containing them and applications to diagnosis and to therapeutic treatment.

Cross-Reference to Related Applications

This application is a divisional of U.S. Patent Application Serial No. 09/486,580, filed August 28, 1998; which is a National Stage Application based on International Patent Application No. PCT/FR98/01864, filed August 28, 1998; said PCT/FR98/01864 claims priority to French Patent Application No. FR 97/10823, filed August 29, 1997. The disclosures of each of the above-identified applications are incorporated herein by reference in their entireties, including all figures, tables, and nucleic acid/amino acid sequences.

Background of the Invention

The present invention relates to a novel human polypeptide defensin Def-X, homologous to HNP-4, its genomic DNA and cDNA.

The invention also relates to cloning and expression vectors, and cells transformed with said vectors. The subject of the invention is also the use of said polypeptides as antibiotic, cytotoxic, repair and endocrine regulatory agent and as pesticide as well as cosmetic or pharmaceutical compositions for the treatment of microbial infections, in particular bacterial, fungal and viral infections, or parasitic infections, cancers, of inflammation and of immune deficiency. Finally, the invention comprises diagnostic methods and kits for the determination of a microbial or parasitic infection and of an inflammation, or for screening for predisposition to immune deficiencies or cancer diseases.

Antimicrobial substances are key elements in the defence of multicellular organisms. Among these substances, there are both simple inorganic compounds (hydrogen peroxide, hypochlorous acid, nitric oxide) and complex proteins and peptides. They are present at the first lines of defence, at the surface of the mucous membranes of various organs, in particular in the epithelial cells of the intestine and of the lungs, depending on the species, as well as in the microbicidal organs of phagocytic cells of hematopoietic origin, where they were first identified. Their synthesis *de novo* or their release from storage sites - organelles of the lysosome or cytoplasmic granule type which are capable of storing them in an

inactive or latent form – can be induced rapidly, which makes them particularly important in the early phases of resistance to infections (Martin et al., 1995).

The antimicrobial proteins of less than one hundred aminoacids in size are arbitrarily called antimicrobial peptides. Several families of antimicrobial peptides have been identified, which differ in the presence within them of disulfide bridges, in their aminoacid composition, their structural conformation and in their activity spectrum. The antimicrobial peptides comprising six conserved cysteines form the defensin family. This family is composed of antimicrobial peptides which are present in numerous species, which are abundant and which are about 3-4 kDa (Ganz and Lehrer, 1994). These peptides are formed of 30 to 40 aminoacids, of which six invariant cysteins which form three intramolecular disulfide linkages. They have complex conformation, are amphipathic, rich in beta antiparallel sheets but lack alpha helices (Lehrer and Ganz, 1992). The antimicrobial action of defensins is thought to result from their insertion into the membranes of the target cells, allowing the formation of voltage-dependent channels. White et al. (1995) describe the possible mechanisms of membrane insertion and of formation of multimeric pores by the defensins, which allow the permeabilization of the membranes of the target cells, for example microbial or tumor cells. The crystallographic structure of human neutrophil defensin HNP-3 (see below) has been determined, and a specific mechanism of dimerization of the human neutrophil defensins is in addition suggested. Increased knowledge of this family of peptides and comparison of their sequences and activity spectra will make it possible to better understand these mechanisms and their specificities, as well as the aminoacid residues more particularly involved in these phenomena.

The defensins are divided into three families of peptides which are structurally different: the "conventional" defensins, the beta-defensins and the insect defensins. These families exhibit differences as regards the position of and the distance between the conserved cysteine residues, as well as those of other conserved aminoacids (proline, glycine) (Ganz and Lehrer, 1995).

Human defensins, of the conventional type, come essentially from two sources. They were first identified by peptide purification from neutrophil extracts. Four defensins have thus been isolated: "human neutrophil peptides" HNP-1, HNP-2, HNP-3, and HNP-4. The first three are different products of the same gene (Ganz et Lehrer, 1995). These three peptides represent 99% of the defensin content of the neutrophils, whereas HNP-4 is also present therein, but at concentrations which are

100 times lower. More recently, two human enteric defensins, HD-5 and HD-6, were characterized in the small intestine and more precisely in the Paneth cells (Bevins et al., 1996). While 16 enteric defensin genes have been identified in mice, only these two homologs have been identified in humans (Mallow et al., 1996).

Defensins have an antimicrobial action on a broad spectrum of microorganismes *in vitro* (Martin et al., 1995). This activity spectrum, which is particularly broad, comprises bacteria, Gram-positive and Gram-negative bacteria, several fungi, mycobacteria, parasites including spirochetes and several enveloped viruses including the HSV and HIV viruses. They are also cytotoxic for several categories of normal and malignant cells, including cells resistant to TNF-alpha and to the cytolytic NK factor (Kagan et al., 1994). The large quantity of targets of the defensins and their abundance in blood cells specialized in the immune defence, as well as the dramatic increase in their concentration during severe infections, suggest that these molecules could play an important role in the natural immunity to infections and to cancers. In particular, the increase in the transcription of the defensin genes and the release of cytoplasmic granules containing presynthesized defensins in response to stimuli, contributes to the local antimicrobial response, it being possible for the defensins to participate in the inflammatory reaction, in the repair processes and in endocrine regulation during infection. The hematopoietic defensins could contribute to the phenomenon of lysis of cancer cells, a phenomenon which is mediated by the neutrophils during the antibody-dependent immune response. The precise physiological role of the enteric defensins is not clearly established. They could stem the proliferation of the intraluminal flora or prevent the translocation of bacteria across the intestinal mucosa (Mallow et al., 1996). The abundance of the defensin mRNA in the Paneth cells reinforces the hypothesis that these epithelial cells could play a key role in the immune defence in the intestine. It has moreover been shown that their expression scheme coincides with the appearance of the Paneth cells during embryogenesis. Mallow et al. (1996) have suggested that low levels of expression of enteric defensins in the fetus could be the evidence of an immaturity of a local defence, which would predispose children born prematurely to infections due to intestinal microorganisms.

A defensin concentration corresponding to 10% of the normal level is observed in patients suffering from "specific granule deficiency", a rare disease of the development of the granulocytes. The affected subjects suffer from frequent infections caused by common bacteria (Ganz and Lehrer, 1995).

Biochemically modified defensins are potential prophylactic and therapeutic agents against infections (Ganz and Lehrer, 1995). Research relating to these antimicrobial peptides or other molecules participating in the natural immunity have gained special importance since phenomena of resistance of microorgnisms to traditional antibiotics started to develop (Bevins et al., 1996).

The primary structure of defensins, in particular of human defensins, has been the subject of recent studies (White et al., 1995; Mallow et al., 1996). The conventional defensins comprise 29 to 35 aminoacids, but are derived from precursors – preproteins – comprising 90 to 100 aminoacids. The proteolytic maturation of the human neutrophil defensins to mature peptides is coupled with their despatch to the granulocytes; the function of the propeptide would include the inactivation of the precursor form of defensin and a support for the acquisition of the active conformation of the mature peptide (Martin et al., 1995). The peptide homologies are maximal at the level of the signal peptides, and minimal at the level of the mature peptides, which comprise nevertheless six cysteine residues which are fully conserved. While the conservation of these residues appears to be necessary for the acquisition of secondary structures which are involved in the activity of defensins, the differences in sequences which exist within the very large family of these antimicrobial peptides, in particular at their N-terminal end, but also in other nonconserved regions, appear to be important determinants of their activity spectrum, and of their antimicrobial or cytotoxic efficacy. The identification of novel members of this family of peptides, in particular of human defensins, is therefore necessary for understanding their mechanism of action and their specificity as well as for their use as anti-infectious and/or cytotoxic agents, or for designing variant peptides exhibiting specific spectra and/or of reduced or increased efficacy.

Sparkes et al. (1989) located the gene encoding HNP-1 on chromosome 8, in the 8p23 region. Bevins et al. (1995), and Mallow et al. (1996) lcoated the two genes encoding HD-5 and HD-6 on chromosome 8, more precisely in the 8p21-pter region, a region including the region previously identified as carrying the hematopoietic defensins. The genes encoding the human enteric defensins HD-5 and HD-6 contain two exons, whereas those encoding the hematopoietic defensins contain three of them, the last two exons encoding the prepropeptide, both in humans and in guinea pigs and rabbits (Mallow et al., 1996). Comparison of the genome sequences of the *HD-5* and *HD-6* genes has revealed a very strong

similarity between the noncoding flanking sequences in 5', suggesting that the latter contain the information necessary to the tissue specificity of the expression of these genes; these same regions carry, in addition, many binding sites for transcription factors, including two AP2 sites and six IL6 sites, suggesting pathways for regulating the expression of these genes during inflammatory processes. More generally, the very high degree of similarity between the sequences and the genomic organization of the defensins HNP-l, 2, 3, 4 and HD-5 and 6 led Bevins et al. (1995) to an evolutionary model attempting to relate the chromosomal organization of the family, and the homologous fractions of each pair of genes.

It is finally advantageous to note that the chromosomal region 8p23 is involved in numerous pathologies, in particular cancer pathologies: there may be mentioned, for example, hepatocellular carcinoma (Becker et al., 1996), non-small cell lung cancer (Sundareshan and Augustus, 1996), prostate cancer (Ichikawa et al., 1996), and colorectal carcinome (Yaremko et al., 1994). Although this has never been documented, it is possible that a deficiency in either of the human defensins has a role in the predisposition to such pathologies, or in their development.

The present invention relates to a novel human defensin, Def-X, which is homologous to defensin HNP-4.

The subject of the present invention is therefore an isolated polypeptide chosen from the following polypeptides:

a) polypeptide whose amino acid sequence is the sequence SEQ ID No. 3;
b) homologous, variant or modified polypeptide of the polypeptide whose amino acid sequence is the sequence SEQ ID No. 3;
c) polypeptide whose amino acid sequence is the amino acid sequence of a biologically active fragment of a polypeptide as defined in a) or b);
d) polypeptide comprising at least one fragment as defined in c).

In the present description, « polypeptide » will also be intended to designate a proteine or a peptide.

According to a preferred embodiment, the polypeptide according to the invention is characterized in that it consists of at least one of the following fragments:

a) signal peptide whose aminoacid sequence is the sequence SEQ ID No. 4, corresponding to the sequence between position 1 and position 19, ends included, of the aminoacid sequence SEQ ID No. 3;

b) proregion whose aminoacid sequence is the sequence SEQ ID No. 5, corresponding to the sequence between position 20 and position 63, ends included, of the aminoacid sequence SEQ ID No. 3;

c) mature peptide whose aminoacid sequence is the sequence SEQ ID No. 6, corresponding to the sequence between position 64 and position 94, ends included, of the aminoacid sequence SEQ ID No. 3; or

d) homologous, variant or modified fragment of a peptide according to a), b) or c).

Still preferably, the polypeptides according to the present invention correspond to the primary structure of the mature defensin defined above, that is to say the structure corresponding to the following aminoacid sequence SEQ ID No. 6:

Ile Cys His Cys Arg Val Leu Tyr Cys Ile Phe Gly Glu His Leu Gly Gly Thr Cys

Phe Ile Leu Gly Glu Arg Tyr Pro Ile Cys Cys Tyr

its homologs, variants or modified forms as well as their biologically active fragments and the polypeptides containing them.

It is clearly understood that the polypeptides of the invention are in a nonnatural form, that is to say that they are not taken in their natural environment but that they may have been obtained by purification from natural sources or obtained by genetic recombination or by chemical synthesis as will be described below.

« Homologous polypeptide» is understood to mean a polypeptide whose aminoacid sequence exhibits at least 80%, and preferably 90%, of aminoacids in common.

« Variant polypeptide » is intended to designate a mutated polypeptide or a polypeptide corresponding to a polymorphism which may exist, in particular in human beings and which may exhibit a truncation, a substitution, a deletion and/or an addition of at least one aminoacid compared with the polypeptide according to the invention.

«Modified polypeptide» is understood to designate a polypeptide obtained by genetic recombination or by chemical synthesis as will be described below, exhibiting a modification relative to the normal sequence. These modifications may in particular apply to the pre, pro- or mature domains of the polypeptide according to the invention, the aminoacids responsible for a specificity of spectrum or of efficacy of activity, or responsible for the structural conformation, the charge or the hydrophobicity, and the multimerization and membrane insertion

of the polypeptide according to the invention. It is thus possible to create polypeptides with equivalent, increased or reduced activity, and with equivalent, lower or broader specificity. The modifications may also apply to the sequences involved in the maturation, transport and addressing of the polypeptide.

«Biologically active fragment» of a polypeptide according to the invention is intended to designate a polypeptide fragment which has conserved at least one activity of the polypeptide from which it is derived, in particular:

- capable of being recognized by an antibody specific for a polypeptide according to the invention; and/or
- capable of acting as an antibiotic; and/or
- capable of acting as an cytotoxic agent; and/or
- capable of acting as an antitumor agent; and/or
- capable of modulating tissue repair, endocrine regulation or the inflammatory process, in particular during an infection.

According to the invention, the biologically active fragments of the polypeptides according to the invention will have a minimum of 10 amino acids, preferably 15 amino acids.

As has been indicated above, among the biologically active fragments, a preferred fragment is the mature peptide having the aminoacid sequence SEQ ID No. 6.

Among the homologs of the mature peptide, there should be mentioned the polypeptides in which up to 5 amino acids have been modified, truncated at the N- or C-terminal end, or deleted, or added, which represents about 80% of the sequence.

The biologically active fragments of this mature peptide preferably comprise from 10 to 15 aminoacids, the advantage of which may be being able to be easily obtained by chemical synthesis.

As indicated, the objective of the modifications of the mature polypeptide will be in particular:

- to modulate the activity of the defensin,
- to modify its specificity, both at the level of the microorganisms on which it is active and on its tissue localization,
- to modify its bioavailability.

The preceding compounds may be obtained using combinatorial chemistry, in which it is possible to systematically vary portions of the polypeptide

before testing them on models, cell cultures or microorganisms, for example, in order to select the compounds which are most active or which have the desired properties.

Chemical synthesis also has the advantage of being able to use:

- nonnatural aminoacids, or
- nonpeptide bonds.

Thus, to enhance the lifespan of the peptides, it may be advantageous to use nonnatural aminoacids, for example in D form, or aminoacid analogs, in particular sulfur-containing forms for example.

Finally, the structure of the mature defensin or of its homologs, variants or modified forms, as well as the corresponding fragments, may be integrated into chemical structures of the polypeptide type and the like. Thus, it may be advantageous to provide at the N- and C-terminal ends compounds not recognized by proteases.

The invention also comprises the nucleic acids encoding a polypeptide according to the invention.

According to a preferred embodiment, the nucleic acids according to the invention will be chosen from the following nucleic acids:

a) nucleic acid having the sequence SEQ ID No. 1 (genomic);
b) nucleic acid having the sequence SEQ ID No. 2 (cDNA);
c) equivalent, homologous, mutated or modified nucleic acid, compared with the nucleic acids according to a) or b);
d) fragments of the sequences a), b) or c) having at least ten bases;
e) nucleic acid capable of hybridizing with one of the sequences as defined in a), b), c) or d).

It is understood that the present invention does not relate to the genomic sequences in their natural chromosomal environment; they are sequences which have been isolated, that is to say that they have been recovered directly or indirectly, their environment having been at least partially modified.

They may thus be genomic DNA, cDNA or RNA comprising nonnatural nucleotides or not; they may also be isolated natural nucleic acids, or synthetic nucleic acids.

Equivalent nucleic acid will be understood to mean a nucleic acid encoding the polypeptides according to the invention, taking into account the degeneracy of the genetic code, and the corresponding cDNAs and RNAs.

Homologous nucleic acids will be understood to mean nucleic acid whose sequence exhibits at least 80%, preferably 90%, homology with the nucleic sequences according to the invention.

Mutated nucleic acid will be understood to mean any nucleic acid encoding a variant polypeptide according to the invention, and any nucleic acid comprising, compared with the sequences SEQ ID No. 1 and SEQ ID No. 2, at least one mutation in the promoter and/or regulatory sequences which may have an effect on the expression of the polypeptide, in particular on its level of expression and the tissue-specificity thereof. The sequences exhibiting a polymorphism which is present in human beings are therefore included in the invention. Among these polymorphisms, some may lead to immune deficiencies, in the response to infections, to predispositions to and/or to the development of cancers.

Modified nucleic acid will be understood to mean any nucleic acid encoding a modified polypeptide according to the invention, or any nucleic acid obtained by mutagenesis according to techniques well known to persons skilled in the art, and comprising modifications relative to the normal sequences, in particular mutations in the regulatory and/or promoter sequences, in particular leading to a modification in the level and/or the tissue-specificity of the expression of the polypeptide.

The present invention relates to all the primers and probes, which may be labeled according to methods well known to persons skilled in the art, which make it possible to identify, in particular by techniques based on hybridization or on amplification, for example by PCR, the nucleic sequences according to the invention, including discriminating between the normal sequences and the mutated sequences. Among the nucleic acid fragments of interest, there should be mentioned in particular the antisense oligonucleotides, that is to say whose structure ensures, by hybridization with the target sequence, inhibition of the expression of the corresponding product. The sense oligonucleotides should also be mentioned which, by interacting with proteins involved in the regulation of the expression of the corresponding product, will induce either an inhibition, or an activation of this expression.

They may be sequences which act both at the level of the exon or intron sequences described and on the flanking sequences, in particular the promoters and/or 5' UTR regions.

The present invention also relates to cloning or expression vectors comprising a nucleotide sequence as described above.

These cloning or expression vectors may comprise elements ensuring expression of the sequence in a host cell, in particular promoter sequences and regulatory sequences which are effective in said cell.

It being possible for the vector in question to be autonomously replicating or to be intended to ensure the integration of the sequence within the chromosomes of the host cell.

In the case of autonomously-replicating systems, depending on the host cell, either prokaryotic or eukaryotic, systems of the plasmid type or viral systems will be preferably used, it being possible for the vector viruses to be in particular advenoviruses (Perricaudet et al., 1992), retroviruses, poxviruses or herpesviruses (Epstein et al., 1992). Persons skilled in the art know the technologies which can be used for each of these viruses.

Thus, it is known to use, as viral vector, defective viruses whose culture is carried out in complementation cells, this avoiding the possible risks of prolifeation of an infectious viral vector.

When the integration of the sequence into the chromosomes of the host cell will be desired, it will be necessary to provide, on either side of the nucleotide sequence to be integrated, one or more sequences derived from the host cell in order to ensure the recombination. These are also methods which are widely described in the prior art. It will be possible, for example, to use systems of the plasmid or viral type; such viruses will be, for example, retroviruses (Temin, 1986) or the AAVs, Adenovirus-Associated Virus (Carter, 1993).

The invention also relates to the prokaryotic or eukaryotic cells transformed with a vector as described above in order to ensure the expression of a natural, normal or variant, or modified defensin Def-X, or for example, of one of its fragments.

As indicated above, the present invention also relates to the polypeptides obtained by culturing the cells thus transformed and recovering the protein expressed, it being possible for said recovery to be carried out intracellularly or extracellularly in the culture medium when the vector has been designed to ensure the secretion of the protein by means, for example, of a "signal" sequence, the polypeptide being in the form of a pre-polypeptide or prepro-polypeptide. The constructs allowing the secretion of the polypeptides are known, both for

prokaryotic systems and for eukaryotic systems. In the context of the present invention, some of the polypeptides Def-X may comprise their own secretory or membrane insertion system.

It is clearly understood that the recombinant polypeptides according to the invention may be obtained in glycosylated or nonglycosylated form and may have the natural tertiary structure or not.

Among the cells which can be used for the production of these polypeptides, there should of course be mentioned bacterial cells (Olins and Lee, 1993), but also yeast cells (Buckholz, 1993), as well as animal cells, in particular mammalian cell cultures (Edwards and Aruffo, 1993) but also insect cells in which methods using baculoviruses, for example (Luckow, 1993), may be used.

The cells thus obtained can make it possible to prepare natural, variant or modified polypeptides, Def-X, but also fragments of these polypeptides, in particular polypeptides which may correspond to the biologically active fragments.

The present invention relates, in addition, to the same polypeptides according to the invention but which are obtained by chemical synthesis and which may comprise nonnatural or modified aminoacids.

The polypeptides according to the present invention, in particular mature defensin, as well as the homologs, derivatives or modified mature polypeptides, may be obtained by chemical synthesis using any one of the numerous peptide syntheses known, for example the techniques using solid phases or techniques using partial solid phases, by condensation of fragments or by a conventional synthesis in solution.

When the compounds according to the present invention are synthesized by the solid phase method, the C-terminal aminoacid is attached to an inert solid support and comprises groups protecting its amino group at the alpha position (and if necessary, protections on its functional side groups).

At the end of this step, the group for protecting the amino terminal group is removed and the second aminoacid, which also comprises the necessary protection, is attached.

The N-terminal protecting groups are removed after each aminoacid has been attached; on the other hand, the protection is of course maintained on the side chains.

When the polypeptide chain is complete, the peptide is cleaved from its support and the protective side groups are removed.

The solid phase synthesis technique is described in particular in Stewart et al. (1984) and Bodanszky (1984).

The details of the synthesis will not be mentioned here; it should simply be recalled that the protective groups which are preferred for the alpha-amino groups are protective groups of the urethane type (BOC or FMOC). As regards the coupling reagents, they are very numerous; among them, there should of course be mentioned more particularly N,N'-diisopropylcarbodiimine (DIC) which is used in general in DMF or DCM.

When it will be desirable to use nonnatural aminoacids, it may be necessary to provide other types of reagent and in particular other types of protection system.

The present invention also relates to polyclonal or monoclonal antibodies obtained by immunological reaction in a human or animal body with an immunogenic agent consisting of a polypeptide according to the invention, in particular a polypeptide obtained by culturing one of the cells which have just been described, or by chemical synthesis as indicated above.

The invention therefore extends to the monoclonal and polyclonal antibodies or one of their fragments, chimeric antibodies, which are capable of specifically recognizing a polypeptide according to the invention.

The invention also comprises the antibodies according to the invention, characterized in that they labeled.

The labeled antibodies may be, for example, immunoconjugated with enzymes such as peroxidase or alkaline phosphatase, or labeled with the aid of fluorescent compounds, biotin or radiolabeled. The labeling techniques are well known to persons skilled in the art and will not be developed in the present description.

The invention also extends to the use of a polypeptide according to the invention as antimicrobial, in particular antibacterial, antifungal, antiviral and/or antiparasitic agent, as cytotoxic agent, in particular for anticancer use, and/or as agent for modulating inflammatory, tissue repair and endocrine, in particular corticostatic, regulating processes.

According to another aspect, the invention relates to a pharmaceutical composition comprising a polypeptide according to the invention, which may be combined with a pharmaceutically acceptable vehicle.

Such a composition may be administered by the systemic, local or topical route.

Its mode of administration, its dosage, its optimal galenic forms may be determined according to the criteria generally taken into account in establishing a treatment appropriate for a patient, in particular their age, their body weight, tolerance of treatment, its observed side effects, and the like.

The invention also comprises a pharmaceutical composition comprising a vector according to the invention which is capable of expressing *in vivo* a polypeptide according to the invention, which may be combined with a pharmaceutically acceptable vehicle.

It is also possible to envisage the expression of polypeptides or their fragments *in vivo*, in particular by means of gene therapy and using the vectors which were described above.

In the context of gene therapy, it is also possible to envisage the use of the sequences of the genes or of the cDNAs described above, « naked », this technique was in particular developed by the company Vical, which has shown that it was, under these conditions, possible to express the polypeptide in some tissues without having recourse to the support of a viral vector in particular.

Still in the context of gene therapy, it is also possible to envisage the use of cells transformed ex vivo, which cells may then be reimplanted, either as such, or inside systems of the organoid type, as is also known in the state of the art (Danos et al., 1993). It is also possible to envisage the use of agents facilitating the targeting of a defined cell type, penetration into the cells or transport to the nucleus.

Said pharmaceutical compositions are, according to the invention, intended for the prevention and/or treatment of microbial infections, in particular microbial infections of bacterial, Gram-positive or Gram-negative bacteria, mycobacterial, fungal and viral origin, or parasitic, in particular spirochet, infections.

According to a preferred embodiment, the invention advantageously relates to the pharmaceutical compositions according to the invention, characterized in that the viral infections are infections linked to enveloped viruses, in particular the HSV and HIV viruses.

The subject of the invention is also pharmaceutical compositions according to the invention, intended for the prevention and/or treatment of cancers, in particular melanomas, liver, prostate or non-small cell lung cancer or colorectal carcinoma.

The invention comprises, in addition, pharmaceutical compositions according to the invention, intended to increase the immune defenses, to increase the immune defenses in the case of acquired immunodeficiency or to prevent the immunodeficiency, in particular for the treatment of psoriasis, or to modulate the inflammatory processes in cases in particular of chronic inflammatory diseases.

The polypeptides according to the present invention can be more particularly used in external topical form, for example on the skin and the mucous membranes. These external topical forms may be for pharmaceutical, dermatological or cosmetic use.

In particular, these compositions may be used as pharmaceutical antiseptic agent or as antiseptic in some cosmetics, either for cleansing the skin or superficial body growths and/or as preservative for the compositions.

The topical compositions according to the present invention may be used in particular in some skin, eye, vaginal or buccal conditions. They may also be used as additional cosmetic agent, in particular in some treatment shampoos.

The invention also relates to the detection of the absence or of an abnormal quantity of protein or of nucleic acid corresponding to defensin X as marker of an infection or of pathologies which will be described below.

The invention also relates to the detection of an abnormal form of the protein or the presence of an abnormal nucleic acid corresponding to a mutated defensin which may possibly be completely inactive. In this case, the presence of this abnormal form may be a marker of predisposition to certain conditions, in particular immunodeficiency and/or cancers.

Accordingly, the present invention relates to a method of diagnosing of an immunodeficiency and/or of a predisposition to certain types of cancer, characterized in that the presence of an abnormal defensin and/or of a sequence encoding an abnormal defensin is detected in a sample from a patient.

The diagnostic methods according to the present invention allow in particular the detection of an immunodeficiency and/or of a predisposition to one or more cancers, in particular those cited above, in particular in at-risk families. This type of diagnosis will in general be carried out by the detection of the mutated forms of the protein or of the nucleic acid sequences.

However, the invention also relates to methods for the diagnosis of inflammation, immunodeficiency, predisposition to conditions of the cancer type and/or infections due to microorganisms or linked to an immune deficiency or inflammatory phenomenon, characterized in that they comprise assaying a polypeptide or a nucleic acid according to the invention in a biological sample and

comparing the result of said assay which is obtained with the quantity of polypeptide or nucleic acid normally present in an equivalent biological sample.

In this case, the peptide assay will allow, in general, detection of a microbial or parasitic infection and/or of an inflammation. The peptide assays may be carried out by any known method, ELISA or RIA for example. The detection of an abnormal form of defensin-X may be carried out, for example, with the aid of a monoclonal antibody which is specific for this form, in particular the antibodies which are the subject of the invention.

According to a preferred embodiment, the invention advantageously comprises the methods characterized in that they use an oligonucleotide probe and/or primer according to the invention.

The methods in which all or part of the sequence corresponding to the polypeptide Def-X is amplified beforehand by assaying nucleic acid according to the invention will be generally preferred, it being possible for these amplification methods to be carried out by the so-called PCR or PCR-like methods. PCR-like will be understood to designate all the methods using direct or indirect reproductions of the nucleic acid sequences, or in which the labeling systems have been amplified, these techniques are of course known; in general, they involve the amplification of DNA by a polymerase; when the original sample is an RNA, it is advisable to carry out a reverse transcription beforehand. There are currently very numerous methods which allow this amplification, for example the so-called NASBA "Nucleic Acid Sequence Based Amplification" (Compton, 1991), TAS "Transcription based Amplification System" (Guatelli et al., 1990), LCR "Ligase Chain Reaction" (Landegren et al., 1988), "Endo Run Amplification" (ERA), "Cycling Probe Reaction" (CPR), and SDA "Strand Displacement Amplification" (Walker et al., 1992), methods which are well known to persons skilled in the art.

The invention relates, in addition, to diagnostic kits or boxes for the determination of a microbial or parasitic infection, an inflammation, an immunodeficiency and/or a predisposition to cancer-type conditions, characterized in that they comprise an antibody according to the invention.

The diagnostic kits or boxes for the determination of a microbial or parasitic infection, an inflammation, an immunodeficiency and/or predisposition to cancer-type conditions, characterized in that they comprise a probe and/or a primer according to the invention are also included in the invention.

Finally, the subject of the invention is the use of a polypeptide according to the invention as pesticide, in particular for the cultivation of plants of industrial

interest such as, for example, food plants such as corn, wheat, soybean, rice or rape, fodder plants, fruit trees, grape vine or ornamental plants.

Other characteristics and advantages of the present invention will emerge on reading the examples below, illustrated by the figures whose legends are described below.

Legend to the figures

Figure 1

Genomic sequence of hDef-X.

Presented is the entire genomic DNA sequence of hDef-X which exhibits significant homology with the gene encoding hDef-4 (HNP-4).

The sequence has the following sites, the presence of which is deduced by homology with the hDef-4 sequence:

• CAAT box	1711-1714
• TATA box	1758-1767
• mRNA start	1836
• exon 1	1836-1874
• splicing site 1	GTCAGT
• Alu insertion	2155-2335
• L1 fragment insertion	2710-2780
• splicing site 2	CAG
• exon 2	3394-3577
• start of coding phase	3406
• splicing site 3	GTGAGA
• splicing site 4	CAG
• exon 3	4164-4379
• end of coding phase	4276
• polyadenylation site	4374-4379.

Figure 2

Alignment of the genomic sequences of the human defensins Def-X and Def-4 (HNP-4).

Alignment of the entire genomic DNA sequence of the novel defensin Def-X exhibiting homology with the genomic DNA of hDef-4 (GenBank accession number U18745).

The annotations present the positions on the hDef-4 sequence of the signals CAAT box, TATA box, splicing sites, beginning and ends of introns/exons, start of transcription and polyadenylation site.

Figure 3

Alignment of the cDNA sequences of hDef-4 (HNP-4) and hDef-X.

The sequences exhibit an overall homology of 61.4%. The alignment reveals an insert of about 75 bases downstream of a STOP codon, which are present on the sequence of hDef-4, but not on that of hDef-X; the strong homology continues on the whole region between the end of this insert and that of the cDNA. Outside this

insertion region, the degree of homology between nucleic sequences is therefore remarkable.

Figure 4

Peptide sequence of the protein hDef-X.

The position of the sites of cleavage of the signal peptide and of the pro region were deduced from the alignment of the peptide sequences of hDef-4 and hDef-X.

Figure 5

Alignment of the peptide sequences of the known human defensins hDef-1, hDef-4, hDef-5, and hDef-6 with hDef-X.

* The star indicates an amino acid which is conserved on the five sequences.

• The dot indicates an amino acid whose class is conserved on the five sequences (amino acid which is either identical, or which is the subject of a conservative substitution).

^ six arrows indicate the positions of the six cysteines conserved across the class of conventional defensins and responsible for the three-dimensional structure necessary for the activity of these peptides.

EXAMPLES

Example 1: Identification of the gene encoding hDef-X

Isolation of BAC B0725B12

To analyze the 8p23 region of the human genome, in particular in the region known to carry genes encoding human defensins, a BAC ("Bacterial Artificial Chromosome") corresponding to said region was isolated. A BAC library covering the complete human genome was prepared from the ADN of a human lymphoblastic line derived from individual No. 8445 of the CEPH families. This line was used as source of high-molecular weight DNA. The DNA was partially digested with the restriction enzyme BamHI, and then cloned at the BamHI site of the plasmid pBeloBacII. The clones thus obtained were "pooled" and screened according to the three-dimensional analytical procedure previously described for the screening of YAC ("Yeast Artificial Chromosome") libraries (Chumakov et al., 1992 and 1995). The three-dimensional pools obtained were screened by PCR with the aid of the primers flanking the marker SHGC-10793, for Neutrophil defensin 4 precursor (GeneBank: accession number U18745); a clone of BAC B0725 B12 was thus isolated.

After digestion with the restriction enzyme NotI, the size of the insert carried by this BAC was determined on a 0.8% agarose gel after migration by

alternating field electrophoreses (CHEF) (4 hours at 9 volts/cm, with an angle of 100°, at 11°C in 0.5 × TAE buffer). It was thus demonstrated that BAC B07025B12 carries an insert of 220 kb, with an internal site for the enzyme NotI.

Chromosomal location of BAC B0725B12 by fluorescent in situ hybridization (FISH)

The chromosomal location of BAC in the candidate region 8p23.1-23.2 was confirmed by fluorescent in situ hybridization (FISH) on metaphase chromosomes, according to the method described by Cherif et al., (1990).

Sequencing of the BAC B0725B12 insert

To sequence the BAC B0725B12 insert, a subclone library was prepared from the sonicated DNA of this BAC.

The cells derived from one liter of "overnight" culture were treated by alkaline lysis according to conventional techniques. After centrifugation of the product obtained in a cesium chloride gradient, 12 µg of BAC B0725B12 DNA were purified. 3 µg of DNA were sonicated in order to obtain fragments whose sizes are uniformly distributed from 1.2 kb to 1.5 kb. The fragments obtained were treated in a volume of 50 µl with 2 units of Vent polymerase for 20 minutes at 70°C, in the presence of the 4 deoxytriphosphates (100 µM). The fragments with blocked ends resulting from this step were separated by electrophoreses on a 1% low-melting point agarose gel (60 volts for 3 hours). The fragments grouped according to their sizes were excised and the bands obtained treated with agarose. After extraction with chloroform and dialysis on Microcon 100 columns, the DNA in solution was adjusted to a concentration of 100 ng/µl. A ligation was performed "overnight" by bringing 100 ng of fragmented DNA of BAC B0725B12 into contact with 20 ng of DNA of the vector BluescriptSK linearized by enzymatic digestion, and treated with alkaline phosphatase. This reaction was carried out in a final volume of 10 µl in the presence of 40 units/µl of T4 DNA ligase (New England Biolabs). The ligation products then served to transform, by electroporation either a strain XL-Blue (for the multicopy plasmids), or a D10HB strain (for the subclones derived from BAC). The $lacZ^-$ clones which are resistant to the antibiotic were subcultured individually in microplates for storage and sequencing.

960 subclones corresponding to the insertion of fragments of 1.2 kb to 1.5 kb at the BamHI site (made blunt) of the plasmid BluescriptSK.

The inserts of these subclones were amplified by PCR on bacterial cultures performed "overnight", using the vector primers flanking the inserts. The sequence of the ends of these inserts (average 500 bases on each side) was

determined by automated fluorescent sequencing on an ABI 377 sequencer equipped with the ABI Prism DNA Sequencing Analysis software (version 2.1.2).

The fragments having a sequence obtained from the sub-BACs were assembled by the Gap4 software of R. Staden (Bonfield et al., 1995). This software allows the reconstruction of a complete sequence from fragments of sequences. The sequence deduced from the alignment of the different fragments is the consensus sequence.

Finally, directed sequencing techniques (systematic primer walking) were used to perfect the sequences and to link the contigs.

Analysis of the sequences for the identification of genes

The potential exons of the insert of BAC B0725B12 were located by searching for homology on public protein, nucleic acid and EST (Expressed Sequence Tag) banks.

Databanks

Local refusions of the principal public banks were used. The protein bank used consists of the nonredundant fusion of the libraries Genpept (automatic translation of GenBank, NCBI; Benson et al., 1996); Swissprot (George et al., 1996) and PIR/NBRF (Bairoch et al., 1996). The doublets were eliminated by the "nrdb" software (public domain, NCBI; Benson et al., 1996). The internal repeats were then masked by the "xnu" software (public domain, NCBI; Benson et al., 1996). The resulting bank, called NRPU (Non-Redundant Protein-Unique) served as reference for the searches for protein homologies. The homologies found with this bank made it possible to locate regions potentially encoding a protein fragment which is at least related to a known protein (coding exons). The EST bank used is composed of the subsections "gbest" (1-9) of Genbank (NCBI; Benson et al., 1996). It contains all the fragments of public transcripts.

The homologies found with this bank made it possible to locate potentially transcribed regions (present on the messenger RNA).

The nucleic acid bank (other than the ESTs) used contains all other subsections of Genbank and of EMBL (Rodriguez-Tome et al., 1996) whose doublets were eliminated as above.

Softwares

All the BLAST softwares (Altschul et al., 1990) for searching for homologies between a sequence and protein or nucleic databanks were used. The significance levels used depend on the length and on the complexity of the region

tested as well as on the size of the reference bank. They were adjusted and adapted to each analysis.

Example 2: analysis of the nucleic and peptide sequences of hDef-X

Structure of the gene encoding hDef-X

The alignment of the gene encoding hDef-X with those encoding the known defensins made it possible to note a maximum homology between hDef-X and hDef-4 (Figure 2). The overall level of homology between the two nucleic sequences is 72%. The only two regions of the genomic DNA of hDef-X which do not exhibit homology with that of hDef-4 correspond to two regions of insertion of a sequence which is repeated in the sequence of hDef-X, which are absent from the sequence of hDef-4: one element of the Alu type (positions 2155 to 2335) and one fragment of element of Line 1 (positions 2710 to 2780).

A high conservation of the region flanking in 5' the promoter region is noted, from which a high conservation of the elements for regulating the stability of the messenger and the expression of the gene probably results.

The high conservation of the sequence of exon 1, which is not translated, makes it possible to definitively attach the defensin hDef-X to the class of hematopoietic conventional defensins, that is hDef-1, 2, 3 and 4, in contrast to the enteric defensins hDef-5 and 6, whose genomic sequence comprises only two exons, both of which are coding.

The alignment of the cDNAs for hDef-4 and hDef-X, indicating a homology greater than 60%, is presented in Figure 3.

Protein analysis

The peptide sequence of the defensin according to the invention is represented in Figure 4. The three domains of the protein are positioned as follows:

- signal peptide: aa 1-19
- pro region: aa 20-63
- mature peptide: aa 64-94.

The specific degrees of homologies between hDef-4 and hDef-X were calculated, according to the relevant region of the protein:

- signal peptide: 63.2 %
- pro region: 52.3 %
- mature peptide: 37.9 %.

The overall homology is 49.5%. These figures confirm the very high homology which exists between defensins, a homology which is maximum at the level of the signal peptides and minimum at the level of the mature peptides.

The amino acids conserved in the class of conventional defensins are found in the primary protein sequence of Def-X, in particular the six cysteines involved in the three-dimensional structure thereof (Figure 5).

In order to predict the secondary structures present on the defensin according to the invention, softwares for predicting secondary structure which are included in the Protein Interpretation Package, Copyright MRC 1994, Medical Research Council, Hillsroad, Cambridge, United Kingdom, were used.

These softwares made it possible in particular to compare the predicted structures of Def-X and HNP-4. Hydrophobicity profiles, alpha-helix structures, β sheets, amphiphilicity are superposable in the two peptides, which suggests similar processes for membrane insertion and for formation of multimeric ion channels for these two defensins.

Example 3: Search for mutations associated with familial cancer cases

Extraction of the genomic DNA

The genomic DNA of immunodeficient or cancer patients is extracted from the peripheral venous blood after cellular lysis, protein digestion, organic partition and finally alcohol precipitation, according to conventional techniques well known to persons skilled in the art.

It is in particular advantageous to study the presence of mutations in the genomic DNA of individuals coming from families with a high rate of cancer, all types of cancer combined. A deficiency in a gene for granulocyte defensin, such as hDef-X, can in fact have a role in predisposition to cancers, as mentioned above.

Amplification of the genomic DNA

Oligonucleotide primers are used for the genomic amplification of the exon sequences derived from BAC B0725B12; they are predicted by computer analysis, and defined with the aid of the OSP software (Hillier et al., 1991).

All these primers contain, upstream of the bases specifically targeted by the amplification, a common universal oligonucleotide tail intended to allow the sequencing of the amplified fragments (PU: 5'-TGTAAAACGACGGCCAGT-3' for the upstream primers, and RP: 5'-CAGGAAACAGCTATGACC-3' for the downstream primers).

The oligonucleotide primers are synthesized according to the phosphoramidite method, on a GENSET UFPS 24.1 synthesizer.

The amplification of each predicted exon sequence is carried out by polymerase chain reaction (PCR), under the following conditions:

Final volume	50 µl
Genomic DNA	100 ng
MgCl2	2 mM
dNTP (for each)	200 µM
Primer (for each)	7.5 pmoles
AmpliTaq Gold DNA polymerase (Perkin)	1 unit
PCR buffer (10X = 0.1 M Tris HCl pH 8.3, 0.5 M KCl)	1 X.

The amplification is carried out in a Perkin Elmer 9600 or MJ Research PTC200 thermocycler with a heating cover. After heating at 94°C for 10 minutes, 35 cycles are performed. Each cycle comprises: 30 seconds at 94°C, 1 minute at 55°C and 30 seconds at 72°C. A final segment of elongation of 7 minutes at 72°C ends the amplification.

The quantity of amplification products obtained is determined on a 96-well microplate, by fluorometry, using the Picogreen intercalating agent (Molecular Probes).

Detection of the polymorphisms/mutations

The products of genomic amplification by PCR are sequenced on the ABI 377 automated sequencer using fluorescent primers labeled with ABI fluorochromes (Joe, Fam, Rox and Tamra) and Thermosequanase DNA polymerase (Amersham).

The reactions are carried out in 96-well microplates, on a Perkin Elmer 9600 thermocycler, under conventional temperature cycle conditions:

- 8 cycles: denaturation: 5 sec. at 94°C; annealing: 10 sec.; extension: 30 sec. at 72°C, and then
- 13 cycles: denaturation: 5 sec. at 94°C; extension: 30 sec. at 72°C.

6 units of Thermosequanase, and 5-25 ng of amplification product are used per sequence reaction.

At the end of the amplification cycles, the products of the sequence reactions are precipitated from ethanol, resuspended in loading buffer containing formamide, denatured, and deposited on 4% acrylamide gels; the electrophoreses (2 hours 30 min at 3000 volts) are conducted on ABI 377 sequencers equipped with ABI softwares for collection and analysis (ABI Prism DNA Sequencing Analysis Software, version 2.1.2.).

The sequences obtained in patients suffering from the deficiencies studied, in particular in patients from families with a high predisposition to cancers,

are compared with the sequences obtained in control subjects, related or not related. A statistical analysis (lod score calculation) makes it possible to conclude as to the significance of the presence of a site of heterozygosity and to its association with a predisposition to cancers.

Example 4: Search for point mutations

The point mutations identified as indicated above can then be detected in patients having a potential deficiency in the gene encoding hDef-X, according to numerous methods known to persons skilled in the art. Among these, the following nonexhaustive lists may be mentioned:

- sequencing
- « single nucleotide primer extension » (Syvanen et al., 1990)
- RFLP
- search for « single strand conformation polymorphism »
- methods based on a cleavage of the mismatched regions (enzymatic cleavage with S1 nuclease, chemical cleavage with various compounds such as piperidine or osmium tetroxide)
- detection of heteroduplex in electrophoresis
- methods based on the use of « allele specific oligonucleotide » (ASO, Stoneking et al., 1991)
- OLA method (« dual color oligonucleotide ligation assay, Samiotaki et al., 1994)
- ARMS (« amplification refractory mutation system »), or ASA (« allele specific amplification »), or PASA (« PCR amplification of specific allele ») (Wu et al., 1989), method.

REFERENCES

Altschul, Stephen F., Gish W., Miller W., Myers E. W., & Lipman D.J. Basic local alignment search tool. J. Mol. Biol. 215:403-10 (1990).

Bairoch A. & Apweiler R. The SWISS-PROT protein sequence data bank and its new supplement TREMBL. Nucleic Acids Res. 24: 21-25 (1996).

Becker S.A., Zou, Y.Z. & Slagle, B.L. Frequent loss of chromosome 8p in hepatitis B virus-positive hepatocellular carcinomas from China. Cancer Res. 56 (21): 5092-7 (1996).

Benson D. A., Boguski M., Lipman D. J. & Ostell J. GenBank. Nucleic Acids Res. 24: 1-5 (1996).

Bodansky M., Principles of peptide synthesis, (1984).

Bevins, C.L., Jones, D.E., Dutra, A., Schaffzin, J. & Muenke, M. Human enteric defensin genes: chromosomal map position and a model for possible evolutionary relationships. Genomics 31: 95-106 (1996).

Bonfield J. K., Smith K. F. & Staden R. A new DNA sequence assembly program. Nucleic Acids Res. 23: 4992-9 (1995).

Buckholz R.G. Yeast Systems for the Expression of Heterologous Gene Products. Curr. Op. Biotechnology 4: 538-542 (1993).

Carter B.J. Adeno-Associated virus vectors. Curr. Op. Biotechnology 3: 533-539 (1993).

Cherif D., Julier C., Delattre O., Derré J., Lathrop G.M., & Berger R.: Simultaneous localization of cosmids and chromosome R-banding by fluorescence microscopy - Applications to regional mapping of chromosome 11. Proc.Natl.Acad.Sci. USA. 87: 6639-6643 (1990).

Chumakov I., Rigault P., Guillou S., Ougen P., Billault A., Guasconi G., Gervy P., Le Gall I., Soularue P., Grinas P. et al. Continuum of overlapping clones spanning the entire human chromosome 21q. Nature 359: 380-386 (1992).

Chumakov I.M., Rignault P., Le Gall I. et al. A YAC contig map of the human genome. Nature 377 supplt: 175-183 (1995).

Compton J. Nucleic Acid Sequence-Based Amplification. Nature 350: 91-92 (1991).

Danos O., Moullier P. & Heard J.M. Réimplantation de cellules génétiquement modifiées dans des néo-organes vascularisés [Reimplantation of genetically modified cells into vascularized neo-organs]. Médecine/Sciences 9:62-64 (1993).

Edwards C.P. et Aruffo A. Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4: 558-563 (1993).

Epstein A.: Les vecteurs herpétiques pour le transfert de gènes [Herpetic vectors for gene transfer] - Médecine/Sciences 8: 902-911 (1992).

Ganz T. & Lehrer R.I. Defensins. Curr. Op. Immunology. 6: 584-9 (1994).

Ganz T. & Lehrer R.I. Defensins. Pharmac. Ther. Vol. 66: 191-205 (1995).

George D. G., Barker W. C., Mewes H. W, Pfeiffer F. & Tsugita A. The PIR-International Protein Sequence Database. Nucleic Acids Res. 24: 17-20 (1996).

Guatelli J.C. et al. Isothermal in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc. Natl. Acad. Sci. USA 87: 1874-1878 (1990).

Hillier L. & Green P. OSP: a computer program for choosing PCR and DNA sequencing primers. PCR Methods Appl. 1: 124-8 (1991).

Ichikawa, T., Nihei, N., Kuramochi, H., Kawana, Y., Killary, A.M., Rinker-Schaeffer, C.W., Barrett, J.C., Isaacs, J.T., Kugoh, H., Oshimura, M. & Shimazaki, J. Metastasis suppressor genes for prostate cancer. Prostate Suppl. 6: 31-35 (1996).

Kagan, B.L., Ganz, T. & Lehrer, R.I. Defensins: a family of antimicrobial and cytotoxic peptides. Toxicology 87: 131-149 (1994).

Landegren U., Kaiser R., Sanders J. & Hood L.A ligase-mediated gene detection technique. Science241: 1077-1080 (1988).

Lehrer & Ganz. Defensins: endogenous antibiotic peptides from human leukocytes. Ciba Found. Sympo. 171: 276-290 (1992).

Luckow V.A. Baculovirus systems for the expression of human gene products. Curr. Op. Biotechnology 4: 564-572 (1993).

Mallow, E.B., Harris, A., Salzman, N., Russel, J.P., DeBerardinis, R.J., Ruchelli, E., & Bevins, C.L. Human enteric defensins. Gene structure and developmental expression. J. Biol. Chem. 271 (8): 4038-4045 (1996).

Martin, E., Ganz, T. & Lehrer, R.I. Defensins and other endogenous peptide antibiotics of vertebrates. J. Leukocyte Biol. 58: 128-136 (1995).

Olins P.O. et Lee S.C. Recent advances in heterologous gene expression in E. coli. Curr. Op. Biotechnology 4: 520-525 (1993).

Perricaudet M., Stratford-Perricaudet L., & Briand P.: La thérapie génique par adénovirus [Gene therapy using adenoviruses] - La Recherche 23: 471-473 (1992).

Rodriguez-Tome P., Stoehr P. J., Cameron G. N., & Flores T. P. The European Bioinformatics Institute (EBI) databases. Nucleic Acids Res. 24: 6-12 (1996).

Samiotaki M., Kwiatkowksi M., Parik J., & Landegren U. Dual-color detection of DNA sequence variants through ligase-mediated analysis. Genomics 20: 238-242 (1994).

Sparkes, R.S., Kronenberg, M., Heinzmann, C., Daher, K.A., Klisak, I., Ganz, T. & Mohandas, T. Assignment of defensin gene(s) to human chromosome 8p23. Genomics 5 (2): 240-4 (1989).

Stewart, J.M. et Yound, J.D. Solid Phase Peptides Synthesis. Pierce Chem. Company, Rockford, 111, 2ème éd., (1984).

Stoneking M., Hedgecock D., Higuchi R.G., Vigilant L., & Erlich H.A. Population variation of human DNA control region sequences by enzymatic amplification and sequence-specific oligonucleotide probes. Am. J. Hum. Genet. 48: 370-382 (1991).

Sundareshan, T.S. & Augustus, M. Cytogenetics of non-small cell lung cancer: simple technique for obtaining high quality chromosomes by fine needle aspirate cultures. Cancer Genet. Cytogenet. 91 (1): 53-60 (1996).

Syvänen A.C., Aalto-Setala K., Harju L., Kontula K. & Soderlund H. A primer-guided nucleotide incorporation assay in the genotyping of Apo E. Genomics 8: 684-692 (1990).

Temin H.M.: Retrovirus vectors for gene transfer. In Kucherlapati R., ed. Gene Transfer, New York, Plenum Press, 149-187 (1986).

Walker G.T., Fraiser M.S., Schram J.L., Little M.C., Nadeau J.G., & Malinowski D.P. Strand displacement amplification: an isothermal in vitro DNA amplification technique. Nucleic Acids Res. 20: 1691-1696 (1992).

White, S.H., Wimley, W.C. & Selsted, M.E. Structure, function, and membrane integration of defensins. Curr. Op. Structural Biology. 5: 521-527 (1995).

Wu D.Y., Ugozzoli L., Pal B.K. & Wallace R.B. Allele-specific amplification of b-globin genomic DNA for diagnosis of sickle cell anemia. Proc. Natl. Acad. Sci. USA 86: 2757-2760 (1989).

Yaremko, M.L., Wasylyshyn, M.L., Paulus, K.L., Michelassi, F. & Westbrook, C.A. Deletion mapping reveals two regions of chromosome 8 allele loss in colorectal carcinomas. Genes Chromosomes Cancer. 10 (1): 1-6 (1994).